

Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects

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2-Arachidonoylglycerol (2-AG) and anandamide are endocannabinoids that activate the cannabinoid receptors CB1 and CB2. Endocannabinoid signaling is terminated by enzymatic hydrolysis, a process that for anandamide is mediated by fatty acid amide hydrolase (FAAH), and for 2-AG is thought to involve monoacylglycerol lipase (MAGL). FAAH inhibitors produce a select subset of the behavioral effects observed with CB1 agonists, which suggests a functional segregation of endocannabinoid signaling pathways *in vivo*. Testing this hypothesis, however, requires specific tools to independently block anandamide and 2-AG metabolism. Here, we report a potent and selective inhibitor of MAGL called JZL184 that, upon administration to mice, raises brain 2-AG by eight-fold without altering anandamide. JZL184-treated mice exhibited a broad array of CB1-dependent behavioral effects, including analgesia, hypothermia and hypomotility. These data indicate that 2-AG endogenously modulates several behavioral processes classically associated with the pharmacology of cannabinoids and point to overlapping and unique functions for 2-AG and anandamide *in vivo*.

The cannabinoid receptors CB1 and CB2 are molecular targets for Δ^9 -tetrahydrocannabinol (**1**), the psychoactive component of marijuana¹. Two endogenous ligands, or endocannabinoids, have also been identified: the arachidonate-based lipids anandamide (*N*-arachidonylethanolamine, AEA, **2**) and 2-arachidonoylglycerol (2-AG, **3**)^{2–4}. The endocannabinoid system regulates a range of physiological processes, including appetite⁵, pain sensation⁶, inflammation⁷ and memory^{8,9}, and is the current focus of considerable pharmaceutical interest to treat disorders such as obesity, chronic pain, anxiety and depression¹⁰.

Endocannabinoid signaling is tightly controlled by enzymatic hydrolysis¹¹. The principal AEA-hydrolyzing enzyme is FAAH (ref. 12). Genetic¹³ or pharmacological^{14,15} disruption of FAAH causes elevations in AEA levels throughout the nervous system and periphery, resulting in multiple CB1- and/or CB2-dependent behavioral effects, including reduction in pain sensation, inflammation, anxiety and depression¹⁶. Notably, several of the other well-known behavioral effects of direct CB1 agonists, such as hypothermia and movement disorders, are not observed in FAAH-disrupted animals^{14,17}. These animals also exhibit wild-type levels of 2-AG, which suggests that additional CB1-regulated behavioral processes may be mediated by 2-AG *in vivo*. Uncoupling AEA- and 2-AG-dependent signaling by selective pharmacological blockade of their respective degradative enzymes could directly address this hypothesis.

Several lines of evidence suggest that MAGL is a primary enzyme responsible for hydrolyzing 2-AG in the nervous system. First,

recombinant expression of MAGL reduces receptor-dependent 2-AG accumulation in cortical neurons¹⁸. Second, immunodepletion of MAGL decreases 2-AG hydrolysis activity in rat brain tissue by 50% (ref. 19). Third, a comprehensive survey of serine hydrolases in the mouse brain has ascribed ~85% of total 2-AG hydrolysis activity in this tissue to MAGL (ref. 20). Finally, promiscuous serine hydrolase inhibitors that block MAGL (along with several other enzymes, including FAAH) substantially raise brain 2-AG levels and produce cannabinoid behavioral effects in mice²¹. However, none of these previous studies have specifically examined the role that MAGL plays in hydrolyzing 2-AG *in vivo*. Toward this goal, several MAGL inhibitors have been described^{6,22,23}, but none show the level of potency and specificity required for general use as *in vivo* pharmacological tools. For instance, arguably the most well-characterized MAGL inhibitor, URB602 (**4**), displays a half-maximal inhibitory concentration (IC₅₀) value of ~200 μ M (ref. 23) and is therefore insufficiently potent for systemic administration *in vivo*. Moreover, this compound has recently been reported to exhibit similar potencies for MAGL and FAAH (ref. 24), which raises concerns about its utility to discriminate between 2-AG and AEA degradative pathways. A second MAGL inhibitor, *N*-arachidonylethanolamine (NAM, **5**; ref. 22), exhibits some selectivity for MAGL over FAAH and other serine hydrolases²⁰ and has been shown to potentiate the pharmacological effects of 2-AG *in vivo*²⁵. However, the maleamide group of NAM is a general thiol-reactive electrophile and will therefore likely cause this inhibitor to

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Received 10 September; accepted 31 October; published online 23 November 2008; doi:10.1038/nchembio.129

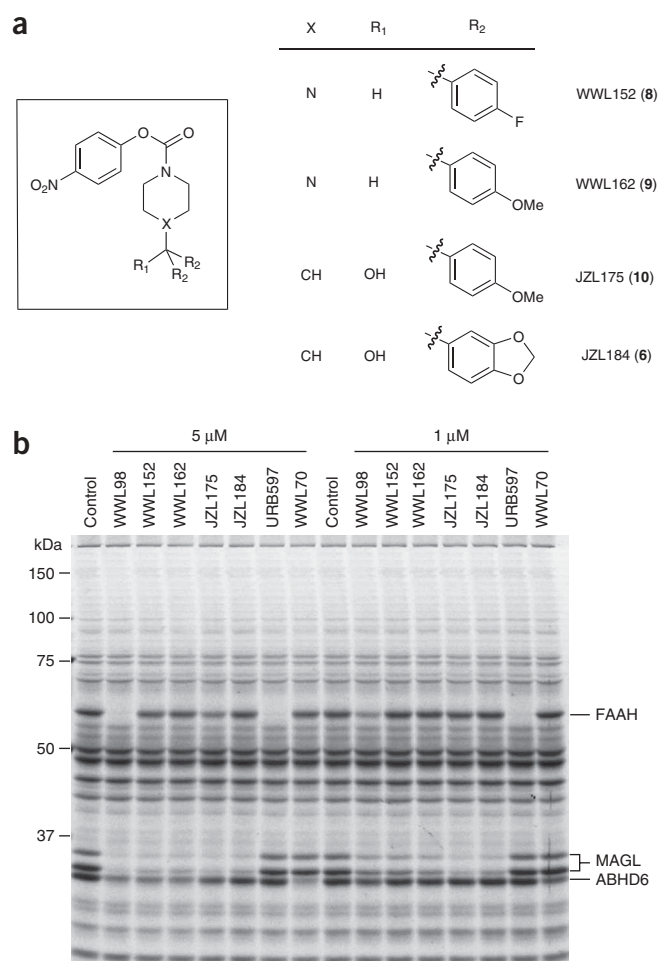


Figure 1 Structures and competitive ABPP profiles of MAGL inhibitors. **(a)** Structures of MAGL inhibitors. **(b)** Competitive ABPP showing the effect of MAGL inhibitors on serine hydrolase activities in the mouse brain membrane proteome. Shown for comparison are the profiles of the selective FAAH and ABHD6 inhibitors, URB597 (ref. 14) and WWL70 (32)²⁷, respectively. Inhibitors were incubated with brain membranes for 30 min, followed by treatment with the serine hydrolase-directed ABPP probe FP-rhodamine (2 μM, 30 min), and the proteomes were then analyzed by SDS-PAGE and in-gel fluorescence scanning to detect inhibited enzymes. Control proteomes were treated with DMSO alone. Fluorescent gel is shown in grayscale. Note that brain MAGL migrates as a 35 kDa doublet by SDS-PAGE, as reported previously^{20,46}.

inactivation by specific chemical groups that show little or no cross-reactivity with other enzymes. Principal among these reactive chemotypes is the carbamate, which has been identified as a privileged scaffold for the design of selective, irreversible inhibitors of serine hydrolases owing to its tempered electrophilicity and hydrolytic stability following covalent reaction (carbamylation) with the conserved serine nucleophile of these enzymes^{26,27}. Second, the functional state of serine hydrolases can be collectively profiled in native biological systems using activity-based protein profiling (ABPP) methods²⁸. ABPP of serine hydrolases uses reporter-tagged fluorophosphonates (FPs), which serve as general activity-based probes for this large and diverse enzyme class²⁹. When performed in a competitive mode, ABPP can serve as a powerful screen to evaluate the potency and selectivity of small-molecule enzyme inhibitors directly in complex proteomes^{30,31}.

In the course of performing competitive ABPP screens with a structurally diverse library of carbamates²⁷, we identified the compound WWL98 (7) (Supplementary Fig. 1 online), which inhibited a specific subset of brain serine hydrolases that included FAAH, MAGL and ABHD6 (Fig. 1b). We next focused on modifying WWL98 to improve potency and selectivity for MAGL. The incorporation of a piperazine spacer and a distal bisaryl motif, as represented in WWL152 (8) and WWL162 (9) (Fig. 1a), was found to preserve potency for MAGL and ABHD6 while greatly reducing activity for FAAH (Fig. 1b). Replacement of the piperazine with a piperidinyl methanol scaffold afforded JZL175 (10) (Fig. 1a), a compound that exhibited selectivity for MAGL over both FAAH and ABHD6 (Fig. 1b). Finally, an additional two-fold enhancement in selectivity was achieved by inclusion of bis(methylene-3,4-dioxyphenyl) groups on the piperidinyl methanol scaffold to provide JZL184 (Fig. 1; also see Supplementary Fig. 1).

JZL184 is a potent and selective MAGL inhibitor

Near-complete blockade of MAGL activity was observed by competitive ABPP following a 30-min preincubation of a mouse brain membrane proteome with as low as 50 nM JZL184 (Fig. 2a). In contrast, inhibition of other enzymes (FAAH and ABHD6) was not observed until much higher concentrations of JZL184 (10 μM) were used. Substrate assays confirmed this degree of selectivity, as JZL184 displayed IC₅₀ values of 8 nM and 4 μM for blockade of 2-AG and oleamide (11) (FAAH substrate) hydrolysis in brain membranes, respectively (Fig. 2b). Comparable inhibitory effects were observed with recombinant MAGL and FAAH when expressed in COS7 cells (Fig. 2c). Notably, brain membranes maintained a residual ~15% 2-AG hydrolysis activity even at the highest concentrations of JZL184 tested, which likely reflects the contribution of other 2-AG hydrolases that are insensitive to JZL184 (ref. 20). JZL184 displayed time-dependent inhibition of MAGL (Supplementary Fig. 2 online), which is indicative of a covalent mechanism of inactivation. We

react with many other cysteine-containing proteins and small molecules (for example, glutathione) *in vivo*. Finally, considering further that several enzymes in addition to MAGL have been shown to hydrolyze 2-AG *in vitro*²⁰, questions remain regarding the specific contribution that MAGL makes to 2-AG signaling *in vivo* and whether blockade of this pathway will produce behavioral effects indicative of heightened endocannabinoid tone.

Here we report the development of a potent and selective MAGL inhibitor, JZL184 (6) (Fig. 1a), that displays robust activity *in vivo*. Key to the success of this pharmacological program was the implementation of activity-based proteomic methods to broadly evaluate and optimize the selectivity of inhibitors across the serine hydrolase superfamily. JZL184 produced a rapid and sustained blockade of brain 2-AG hydrolase activity in mice, resulting in eight-fold elevations in endogenous 2-AG levels that were maintained for at least 8 h. In contrast, AEA levels were unaffected by JZL184. JZL184-treated mice showed a wide array of CB1-dependent behavioral effects, including analgesia, hypomotility and hypothermia, that suggest a broad role for 2-AG-mediated endocannabinoid signaling throughout the mammalian nervous system.

RESULTS

Development of the MAGL inhibitor JZL184

The pursuit of selective inhibitors for serine hydrolases has the potential to benefit from multiple features that are special to this enzyme class. First, serine hydrolases are susceptible to covalent

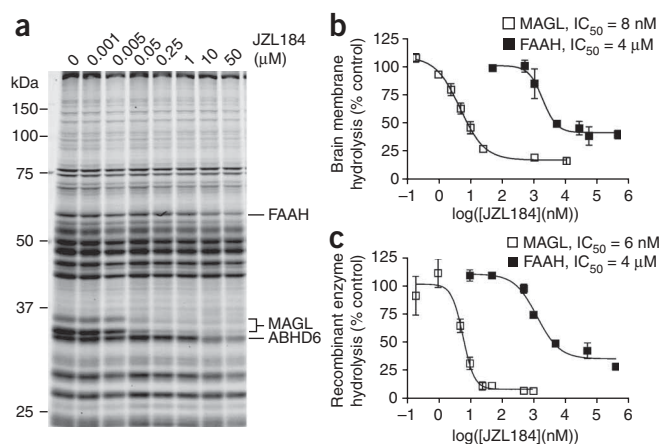


Figure 2 *In vitro* characterization of JZL184. (a) Concentration-dependent effects of JZL184 on mouse brain membrane serine hydrolases as determined by competitive ABPP. (b) Blockade of brain membrane MAGL and FAAH activity by JZL184 as determined with substrate assays (2-AG and oleamide, respectively). (c) Blockade of recombinant MAGL and FAAH activity by JZL184 as determined with substrate assays (2-AG and anandamide, respectively). Enzymes were recombinantly expressed in COS7 cells. Note that JZL184 produced a near-complete blockade of recombinant MAGL activity (>95%), but ~15% residual 2-AG hydrolysis activity was observed in brain membranes, which likely reflects the activity of other enzymes²⁰. For a–c, samples were treated with JZL184 for 30 min before analysis. For b and c, data are presented as means ± s.e.m. for three independent experiments.

therefore also evaluated the relative activity of JZL184 for MAGL and FAAH by measuring $k_{\text{obs}} [I]^{-1}$ values, which confirmed >300-fold selectivity for inhibition of MAGL (4,400 and 13 $\text{M}^{-1} \text{s}^{-1}$, respectively). Finally, we tested JZL184 for activity on other components of the endocannabinoid system and on enzymes involved in arachidonate metabolism. JZL184 did not interact with CB1 or CB2 receptors and did not inhibit the 2-AG biosynthetic enzymes diacylglycerol lipase- α and diacylglycerol lipase- β , or the arachidonic acid-mobilizing enzyme cytosolic phospholipase A₂ group IVA (Supplementary Fig. 3 online). Collectively, these data indicate that JZL184 potently and selectively inactivates MAGL in the mouse brain proteome.

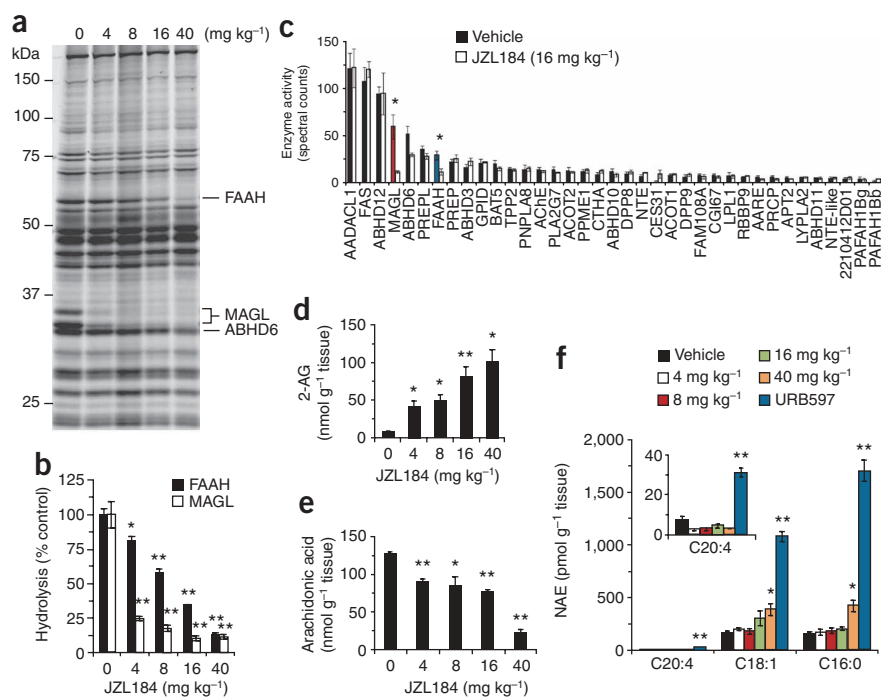
JZL184 inhibits MAGL *in vivo* and elevates 2-AG levels

To assess the ability of JZL184 to block MAGL *in vivo*, male C57Bl/6 mice were administered with JZL184 (4–40 mg kg^{-1} , intraperitoneally) and killed after 4 h for analysis. At the lowest dose of JZL184 tested (4 mg kg^{-1}), competitive ABPP of brain membrane proteomes revealed 75% MAGL inactivation with minimal effects (<20% inhibition) on other brain serine hydrolases, including FAAH (Fig. 3a). These data were also confirmed for MAGL and FAAH by substrate assays (Fig. 3b). Residual brain 2-AG hydrolysis

activity could be further reduced from 25% to 15% of control values by increasing the dose of JZL184 from 4 to 16 mg kg^{-1} (Fig. 3b), which correlated with a near-complete blockade of MAGL activity as determined by competitive ABPP (Fig. 3a). FAAH was also inhibited in a dose-dependent manner, but even at 16 mg kg^{-1} of JZL184, a substantial fraction of FAAH activity (~35%) remained intact as determined by competitive ABPP (Fig. 3a) or substrate (Fig. 3b) assays.

Although our gel-based ABPP analysis already suggested high selectivity for MAGL *in vivo*, the limited resolution of this method precluded a complete assessment of the functional state of brain serine hydrolases in JZL184-treated animals. We therefore examined brain proteomes using an advanced LC-MS platform, termed ABPP-MudPIT, that displays enhanced resolution and sensitivity compared with gel-based ABPP (ref. 32). Briefly, brain membrane proteomes from mice treated with JZL184 or vehicle were subjected to competitive ABPP with the biotinylated FP probe FP-biotin²⁹ (12). FP-biotin-labeled proteins were then enriched with avidin, digested on-bead with trypsin, analyzed by multidimensional LC-MS and identified using the SEQUEST search algorithm. ABPP-MudPIT confirmed that JZL184 (16 mg kg^{-1} , 4 h) produced a near-complete blockade of MAGL activity and partial inhibition of FAAH (Fig. 3c). Notably,

Figure 3 *In vivo* characterization of JZL184. (a,b) Serine hydrolase activity profiles (a) and MAGL and FAAH activities (b) of brain membranes prepared from mice treated with JZL184 at the indicated doses (4–40 mg kg^{-1} , intraperitoneally) for 4 h. (c) ABPP-MudPIT analysis of serine hydrolase activities in brain membranes prepared from mice treated with JZL184 (16 mg kg^{-1} , intraperitoneally, 4 h). MAGL and FAAH control signals are shown in red and blue bars, respectively. (d–f) Brain levels of 2-AG (d), arachidonic acid (e) and NAEs (f) from mice treated with JZL184 at the indicated doses (4–40 mg kg^{-1} , intraperitoneally) for 4 h. For f, data from mice treated with URB597 (10 mg kg^{-1} , intraperitoneally) are also shown, confirming the elevations of NAEs induced by this FAAH inhibitor. For b–f, * $P < 0.05$; ** $P < 0.01$ for inhibitor-treated versus vehicle-treated animals. Data are presented as means ± s.e.m.; $n = 3$ –5 mice per group.



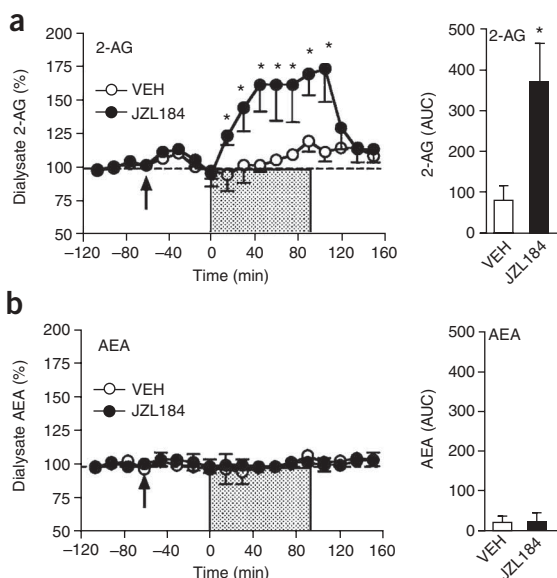


Figure 4 JZL184 raises interstitial levels of 2-AG following neuronal depolarization. Effects of JZL184 (10 mg kg^{-1} , intraperitoneally) on interstitial levels of 2-AG and AEA were measured by *in vivo* microdialysis sampling from the nucleus accumbens of C57Bl/6 mice. Endocannabinoid release was stimulated by neuronal depolarization during perfusion with a high potassium and calcium artificial CSF solution ($t = 0\text{--}90 \text{ min}$; shaded bar). **(a,b)** Depolarization significantly increased dialysate 2-AG levels in both vehicle-treated ($F(10,50) = 2.12$, $P < 0.05$) and JZL184-treated ($F(10,70) = 5.567$, $P < 0.0001$) mice, and this effect was substantially more robust in JZL184-treated animals as demonstrated by analysis of both the temporal profile (pretreatment \times time interaction ($F(10,120) = 3.355$, $*P < 0.001$)) **(a)** and area under the curve (AUC) measures (AUC $t = 0\text{--}150 \text{ min}$; $F(1,12) = 8.737$; $*P < 0.05$) **(b)**. There was no significant alteration in dialysate AEA levels following JZL184 administration and no significant effect of the high potassium and calcium solution on dialysate AEA levels in either group of mice, as determined by analysis of both temporal profile and AUC measures. Data are the mean \pm s.e.m. of the percent change from baseline levels. Baseline dialysate 2-AG levels were $4.6 \pm 0.7 \text{ nM}$ and $4.2 \pm 0.4 \text{ nM}$, and dialysate AEA levels were $0.54 \pm 0.1 \text{ nM}$ versus $0.58 \pm 0.08 \text{ nM}$ for the JZL184 ($n = 8$) and vehicle ($n = 6$) groups, respectively. Pretreatments with JZL184 were administered at $t = -60 \text{ min}$ (denoted by arrow).

none of the other ~ 40 brain serine hydrolases profiled by ABPP-MudPIT were significantly ($P > 0.05$) affected by JZL184 (**Fig. 3c** and **Supplementary Table 1** online). These data demonstrate that, even when administered at a high dose (16 mg kg^{-1}) for an extended period of time (4 h), JZL184 maintains considerable selectivity for MAGL in the brain.

Having established the selectivity profile of JZL184 *in vivo*, we next measured brain levels of candidate endogenous substrates and products for MAGL and FAAH. Even at the lowest dose of JZL184 tested (4 mg kg^{-1}), 2-AG levels were elevated by five-fold at 4 h post-treatment and could be further elevated to eight-fold or ten-fold above baseline at higher doses of inhibitor (**Fig. 3d**). Consistent with previous studies²¹, elevations in brain 2-AG were accompanied by significant ($P < 0.05$) reductions in the levels of arachidonic acid (**13**) (**Fig. 3e**). Other MAGs, such as monopalmitoylglycerol (**14**, PG) and mono-oleoylglycerol (**15**, OG), and their corresponding free fatty acids palmitic acid (**16**) and oleic acid (**17**), respectively, were not significantly ($P > 0.05$) altered in brains from JZL184-treated animals (**Supplementary Fig. 4** online). Importantly, brain anandamide levels were also unaffected by JZL184 at all of the doses tested (**Fig. 3f**). Other *N*-acylethanolamines (NAEs), such as *N*-palmitoylethanolamine (PEA, **18**) and *N*-oleoylethanolamine (OEA, **19**), did not change except at the highest dose of JZL184 tested (40 mg kg^{-1}), where modest (\sim two-fold) elevations in these lipids were observed. The lack of impact of JZL184 on NAE levels is consistent with previous studies showing that partial disruption of FAAH is insufficient to raise NAE levels *in vivo*^{33,34}. The lipid profiles of JZL184-treated mice contrasted sharply with those induced by the selective FAAH inhibitor URB597 (**20**), which completely inhibited brain FAAH activity and caused significant ($P < 0.01$) elevations in anandamide and other NAEs without altering 2-AG or arachidonic

acid levels in this tissue (**Fig. 3f** and **Supplementary Fig. 4**). Similar data were also obtained following oral administration of JZL184 (**Supplementary Fig. 5** online), which indicates that this compound can be administered by multiple routes to achieve selective blockade of MAGL *in vivo*.

To determine whether MAGL inhibition also produced increases in signaling-competent release of extracellular 2-AG, we analyzed JZL184-treated mice by *in vivo* microdialysis following neuronal activation^{35,36}. JZL184 substantially elevated the interstitial brain levels of 2-AG following neuronal depolarization (**Fig. 4a**) but did not affect interstitial brain levels of AEA (**Fig. 4b**). These data indicate that blockade of MAGL by JZL184 elevates both bulk and signaling-dependent pools of 2-AG in the nervous system.

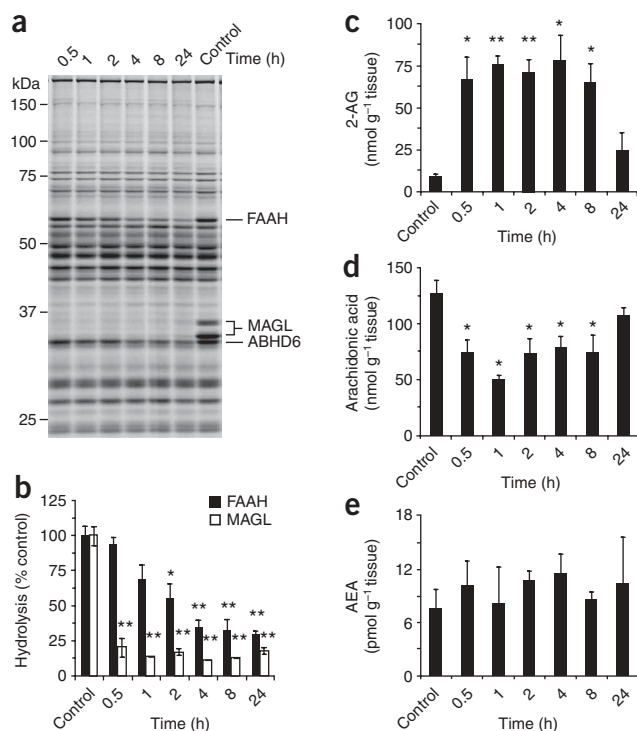


Figure 5 Time course analysis of inhibitory activity of JZL184 *in vivo*. **(a,b)** Serine hydrolase activity profiles **(a)** and MAGL and FAAH activities **(b)** of brain membranes prepared from mice treated with JZL184 (16 mg kg^{-1} , intraperitoneally) for the indicated times. **(c–e)** Brain levels of 2-AG **(c)**, arachidonic acid **(d)** and AEA **(e)** from mice treated with JZL184 (16 mg kg^{-1} , intraperitoneally) for the indicated times. For **b–e**, $*P < 0.05$, $**P < 0.01$ for inhibitor-treated versus vehicle-treated control animals. Data are presented as means \pm s.e.m.; $n = 3\text{--}5$ mice per group.

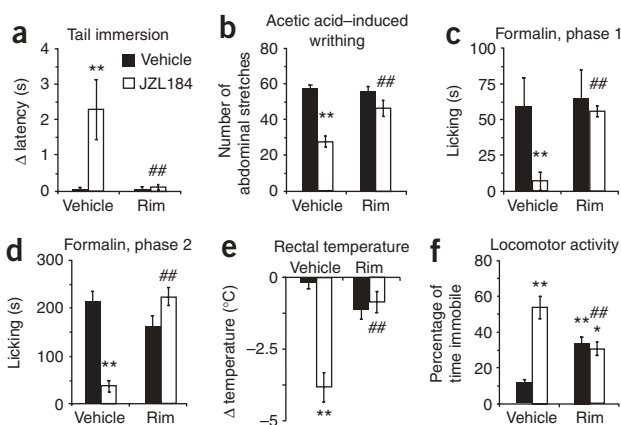


Figure 6 Behavioral effects of JZL184. (a–d) JZL184 produced antinociceptive effects in the tail immersion assay of thermal pain sensation (a; 16 mg kg⁻¹, intraperitoneally) in the acetic acid abdominal stretching assay of noxious chemical pain sensation (b; 16 mg kg⁻¹, intraperitoneally) and in both phase 1 (c; 40 mg kg⁻¹, oral gavage) and phase 2 (d; 40 mg kg⁻¹, oral gavage) of the formalin test. These effects were blocked by pretreatment with the CB1 antagonist rimonabant (Rim, 3 mg kg⁻¹). (e,f) JZL184 (16 mg kg⁻¹ intraperitoneally) also produced significant hypothermia (e) and hypomotility (f) that were significantly attenuated by rimonabant. The baseline tail immersion latency and rectal temperature were 0.82 ± 0.02 s and 37.4 ± 0.1 °C, respectively. **P* < 0.05, ***P* < 0.01 for vehicle-vehicle-treated mice versus vehicle-JZL184-treated mice. ##*P* < 0.01 for vehicle-JZL184-treated mice versus rimonabant-JZL184-treated mice. Data are presented as means ± s.e.m.; *n* = 6–14 mice per group.

Inhibition of MAGL is rapid and persistent in mice

To determine the time course of JZL184 inhibition *in vivo*, mice were administered JZL184 (16 mg kg⁻¹, intraperitoneally) and killed at 0.5, 1, 2, 4, 8 and 24 h for analysis. ABPP of brain membrane proteomes (Fig. 5a) as well as 2-AG hydrolysis assays (Fig. 5b) indicated that MAGL inhibition was rapid, with maximal inhibition achieved within 0.5 h post-treatment, and long-lasting, with >80% inhibition of 2-AG hydrolysis activity persisting for at least 24 h (Fig. 5a). In contrast, FAAH blockade occurred much more slowly and never exceeded 70% at any time point examined (Fig. 5a,b). Concomitant with the rapid inhibition of MAGL, JZL184 treatment caused swift accumulation of 2-AG in the brain. By 0.5 h, brain 2-AG levels were already elevated seven-fold and remained seven-fold to nine-fold above control levels for at least 8 h (Fig. 5c). Notably, 2-AG levels returned to near-control levels by 24 h, even though >80% of the activity of MAGL remained inhibited at this time point. Elevations in 2-AG were matched by a rapid and sustained decrease in arachidonic acid (Fig. 5d). NAEs (including anandamide), as well as other MAGs and free fatty acids, were unchanged throughout the time-course analysis of JZL184-treated mice (Fig. 5e and Supplementary Fig. 6 online).

Behavioral effects of JZL184 in mice

The considerable and sustained elevations in brain 2-AG levels caused by JZL184 suggested that this inhibitor might induce endocannabinoid-mediated behavioral effects. Direct CB1 agonists are known to promote multiple behavioral effects in rodents, including analgesia, hypomotility, hypothermia and catalepsy (collectively referred to as the tetrad test for cannabinoid activity³⁷). FAAH inhibitors are largely inactive in the tetrad test; they cause analgesia but not other cannabinoid behavioral phenotypes¹⁵. JZL184 (16 mg kg⁻¹, 2 h) was also found to exhibit significant (*P* < 0.01) analgesic activity in several pain assays, including the tail-immersion test of acute thermal pain sensation (Fig. 6a), the acetic acid writhing test of visceral pain (Fig. 6b) and the formalin test of noxious chemical pain (Fig. 6c,d). In each case, the effects of JZL184 were blocked by pretreatment with the CB1 antagonist rimonabant (21) (3 mg kg⁻¹) (Fig. 6a–d). In marked contrast to FAAH inhibitors, JZL184 also promoted significant (*P* < 0.01) hypothermia (Fig. 6e) and hypomotility (Fig. 6f). JZL184 treatment did not, however, induce catalepsy when assessed in the bar test. The hypothermic effect of JZL184 was completely blocked by rimonabant (Fig. 6e), which indicates that it is mediated by CB1 receptors. We could not unequivocally determine the contribution of CB1 receptors to the hypomotile effect of JZL184 because rimonabant was found, on its own, to induce

significant (*P* < 0.01) reductions in movement (as has been previously reported³⁸) (Fig. 6f). However, a partial blockade of JZL184-induced immobility was observed with rimonabant (Fig. 6f), which suggests a contribution of CB1 receptors to this phenotype. Finally, JZL184 did not produce hypomotility, hypothermia or analgesia in CB1 receptor-knockout mice (*Cnr1*^{-/-} mice) (Supplementary Fig. 7 online). These data collectively indicate that JZL184 induces a broad array of cannabinoid behavioral effects in rodents that qualitatively mimic much of the pharmacological profile of direct CB1 agonists.

DISCUSSION

Many neurotransmitter systems exhibit diversification at the level of receptors, where multiple ionotropic and/or metabotropic receptors are activated by a single ligand³⁹. The endocannabinoid system is unusual in also having at least two physiological ligands (AEA and 2-AG), thus raising provocative questions about the respective roles that these endocannabinoids play in nervous system function. This problem can be experimentally addressed by perturbing the enzymatic pathways responsible for AEA and 2-AG metabolism. For instance, the genetic¹³ or pharmacological^{14,15} disruption of FAAH has provided evidence that AEA signaling pathways regulate pain, inflammation and neuropsychiatric processes. Equivalent experimental tools to selectively perturb 2-AG metabolism, however, have been lacking. Here, we have described JZL184, a highly efficacious and selective inhibitor of the 2-AG-degrading enzyme MAGL. The observation that brain 2-AG hydrolysis activity was reduced by ~85% following administration of JZL184 to mice provides *in vivo* confirmation of previous *in vitro* estimates of the principal contribution that MAGL makes to total brain 2-AG hydrolysis activity^{20,22}.

Blockade of MAGL activity was sufficient to raise brain 2-AG levels by eight-fold to ten-fold. A similar elevation in stimulated release of 2-AG was observed by *in vivo* microdialysis following inhibition of MAGL. Whether further increases in bulk and/or interstitial levels of 2-AG might occur upon concurrent blockade of additional brain 2-AG hydrolases, such as ABHD6 and ABHD12 (ref. 20), remains unknown. Notably, we did not observe changes in baseline interstitial levels of 2-AG following treatment with JZL184 (Fig. 4a), even though this inhibitor was administered to mice 1 h before neuronal depolarization (a time period that was sufficient to raise bulk brain 2-AG levels; Fig. 5c). These data could indicate that 2-AG is sequestered intracellularly in neurons before depolarization events, which then induce release of this endocannabinoid. The increased brain levels of 2-AG induced by a single dose of JZL184 were maintained for a substantial period of time (≥ 8 h), which likely reflects the relatively long half-life of this inhibitor *in vivo* (~7 h; see Supplementary Fig. 8 online). It is notable, however, that brain 2-AG levels returned to

baseline by 24 h post-treatment with JZL184, even though MAGL remained mostly inactive at this time point. These results might indicate a homeostatic feedback mechanism that operates to rectify heightened 2-AG concentrations in the nervous system. JZL184 did not significantly ($P > 0.05$) alter the levels of NAEs, including AEA, or other monoacylglycerols, including PG and OG. These data indicate a surprisingly selective role for MAGL in regulating 2-AG in the nervous system. Given that MAGL is capable of hydrolyzing other monoacylglycerols *in vitro*, its selective control over 2-AG in the nervous system must reflect other yet-unknown factors. Possibilities include (i) differences in the mechanisms of biosynthesis of distinct classes of monoacylglycerols and the coupling of these pathways to MAGL, and (ii) spatial segregation of MAGL and 2-AG from the other monoacylglycerols.

The rapid and sustained elevations in 2-AG induced by JZL184 were accompanied by an array of CB1-dependent behavioral effects, including analgesia, hypomotility and hypothermia. This collection of phenotypes qualitatively resembles those induced by direct CB1 agonists^{13,37}. However, clear differences were also noted. For instance, JZL184-treated mice did not develop catalepsy and appeared generally normal in terms of their overt posture and appearance (that is, spontaneous limb splaying was not observed). The hypothermic and analgesic effects caused by JZL184 also appeared lower in magnitude relative to those produced by CB1 agonists¹³. Overall, these data suggest that MAGL-regulated 2-AG pathways endogenously modulate several behavioral processes classically associated with the pharmacology of cannabinoids, which contrasts with the more discrete set of phenotypes observed upon disruption of FAAH. These findings could potentially have therapeutic implications. Much of the enthusiasm for FAAH as a potential drug target has stemmed from the lack of overt cannabinoid behavior observed upon disruption of this enzyme^{40,41}. One cannot readily arrive at the same conclusion for MAGL, given that blockade of this enzyme produced CB1-dependent phenotypes, such as hypothermia and hypomotility, that might be viewed as undesirable from a medicinal perspective. It remains possible, however, that these phenotypes could be pharmacologically uncoupled from more beneficial effects (for example, analgesia) by titrating the magnitude of MAGL inhibition *in vivo*. Indeed, we observed significant ($P < 0.05$) increases in 2-AG levels across the entire dose range of JZL184 tested in this study (4–40 mg kg⁻¹), even though the lower doses resulted in less blockade of MAGL activity. These data thus indicate that even partial inhibition of MAGL may be sufficient to augment 2-AG-mediated endocannabinoid signaling *in vivo*.

Looking forward, the chemical scaffold of JZL184 should provide a fertile starting point for medicinal chemistry work to improve the properties of MAGL inhibitors. Regarding selectivity, our ABPP studies suggest that, among the more than 40 serine hydrolases expressed in the mammalian brain, the most common 'off-target' for MAGL inhibitors is likely FAAH. Indeed, despite showing greater than 300-fold selectivity for MAGL over FAAH *in vitro*, JZL184 still partially blocked FAAH activity *in vivo*. This partial inhibition, however, did not result in elevated levels of AEA or of other NAEs, which is consistent with previous findings indicating that >80% blockade of FAAH is required to raise NAEs *in vivo*³⁴. Additional serine hydrolase targets of JZL184 may be found in peripheral tissues, such as liver, where carboxylesterases have been shown to be sensitive to carbamate inhibitors^{26,42}. Moreover, our functional proteomic screen does not exclude the possibility that JZL184 interacts with other proteins outside of the serine hydrolase class, including enzymes (such as oxidoreductases) that may also participate in 2-AG

metabolism^{43,44}. Future studies where the behavioral effects of JZL184 are compared with those observed in mice with a genetic deletion of MAGL should further clarify the specificity of JZL184.

In summary, we believe that the properties of JZL184 warrant inclusion of this compound among the growing arsenal of efficacious and selective pharmacological probes used to examine the endocannabinoid system¹⁰. With selective inhibitors now available for two of the principal endocannabinoid degradative enzymes, FAAH and MAGL, investigators are in a position to experimentally discriminate between the activities of AEA and 2-AG in a wide range of biological systems. Our initial findings, when integrated with previous studies^{14,15}, argue that these endocannabinoids have distinct but overlapping functions *in vivo*. The activity displayed by JZL184 in several components of the tetrad test for cannabinoid pharmacology suggests a potentially broad role for MAGL and 2-AG pathways throughout the nervous system. In contrast, FAAH and AEA pathways may participate in a more restricted set of cannabinoid signaling networks *in vivo*. Finally, it is possible that behavioral processes (such as pain sensation) that are regulated by both AEA and 2-AG (ref. 6) could be even more strongly affected by dual MAGL-FAAH inhibitors. JZL184 could itself serve as a lead scaffold for the development of such dual inhibitors, given that at high concentrations this compound inhibited both MAGL and FAAH without affecting other brain serine hydrolases.

METHODS

Materials. 2-AG, pentadecanoic acid (PDA, **22**), AEA and URB597 were purchased from Cayman Chemicals. Monopentadecanoin (**23**) was purchased from Nu-Chek-Prep, Inc. Tetrahydrolipstatin (THL, **24**) and methylarachidonoylfluorophosphonate (MAFP, **25**) were purchased from Sigma. 1-Palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC, **26**) and 1- α -phosphatidylinositol-4,5-bisphosphate (PIP₂, **27**) were purchased from Avanti Polar Lipids. 1- α -1-Palmitoyl-2-arachidonoylphosphatidylcholine[arachidonoyl-1-¹⁴C] (**28**) was purchased from PerkinElmer. FP-rhodamine (**29**) and FP-biotin were synthesized as described previously^{29,45}. Rimobabant was obtained from the US National Institute on Drug Abuse and dissolved in a vehicle of 18:1:1 v/v/v saline, ethanol and alkamuls-620 (Rhône-Poulenc). ¹³C-Oleamide (¹³C₁₈H₃₅NO) was synthesized from ¹³C-oleic acid (Spectra Stable Isotopes) as previously described¹².

Chemical synthesis of JZL184. JZL184 was synthesized from 4-bromo-1,2-methylenedioxybenzene (**30**) and ethyl *N*-Cbz-isonipecotate (**31**) in three steps and 35% overall yield. ¹H NMR (400 MHz, CDCl₃): δ 1.40–1.48 (m, 2H), 1.62–1.69 (m, 2H), 2.16 (s, 1H), 2.48 (t, $J = 12$ Hz, 1H), 2.88 (t, $J = 13$ Hz, 1H), 3.01 (t, $J = 13$ Hz, 1H), 4.30 (t, $J = 10$ Hz, 2H), 5.93 (s, 4H), 6.75 (d, $J = 9$ Hz, 2H), 6.91–6.93 (m, 4H), 7.26 (d, $J = 9$ Hz, 2H), 8.23 (d, $J = 9$ Hz, 2H); ¹³C NMR (100 MHz, CDCl₃): δ 26.5, 26.9, 44.5, 44.8, 45.2, 79.6, 101.3, 106.9, 108.1, 119.0, 122.5, 125.2, 139.7, 145.0, 146.5, 148.0, 152.3, 156.5; HRMS (m/z): [M+Na]⁺ calcd. for C₂₇H₂₄N₂NaO₉, 543.1374; found, 543.1368. See **Supplementary Methods** online for details on the synthesis of MAGL inhibitors.

Competitive ABPP experiments. *In vitro* inhibitor selectivity was examined using a competitive ABPP method as described previously³⁰. Briefly, mouse brain membrane proteomes, prepared as described in the **Supplementary Methods**, were diluted to 1 mg ml⁻¹ in phosphate-buffered saline (PBS) and pre-incubated with varying concentrations of inhibitors (1 nM to 10 μ M) for 30 min at 37 °C before the addition of FP-rhodamine at a final concentration of 2 μ M in a 50- μ l total reaction volume. After 30 min at 25 °C, the reactions were quenched with 4 \times SDS-PAGE loading buffer, boiled for 5 min at 90 °C, subjected to SDS-PAGE and visualized in-gel using a flatbed fluorescence scanner (Hitachi).

Enzyme activity assays. MAGL and FAAH substrate hydrolysis assays were performed using previously described LC-MS assays²⁰ as detailed in the **Supplementary Methods**.

In vitro studies with JZL184. Standard assays were performed by pre-incubating protein samples with JZL184 for 30 min at 37 °C before the addition of substrate or ABPP probe. Concentration-dependence inhibition curves were obtained from substrate assays and were fit using Prism software (GraphPad) to obtain effector concentration for half-maximum response (EC_{50}) values with 95% confidence intervals. For measurement of k_{obs} [I] $^{-1}$ values, brain membrane proteomes (1 mg ml $^{-1}$, 300 μ l total) were incubated with JZL184 (0.01–15 μ M, 10–40 min, 37 °C). Every 10 min, 50 μ l of the reaction was removed and treated with FP-rhodamine (2 μ M) for 2 min, quenched with 4 \times SDS-PAGE loading buffer and boiled for 5 min at 90 °C. The combined reactions were subjected to SDS-PAGE and visualized in-gel using a flatbed fluorescence scanner. The percentage activity remaining was determined by measuring the integrated optical density corresponding to the MAGL or FAAH bands, and the results were fit to an exponential curve to determine the pseudo-first-order rate constants.

In vivo studies with JZL184. JZL184 (neat) was dissolved by vortexing, sonicating and gentle heating directly into 4:1 v/v PEG300/Tween80 (10, 4, 2 or 1 mg ml $^{-1}$). Male C57Bl/6J mice (6–8 weeks old, 20–26 g) were intraperitoneally administered JZL184 or a 4:1 v/v PEG300/Tween80 vehicle without JZL184 at a volume of 4 μ l g $^{-1}$ weight (40, 16, 8 or 4 mg kg $^{-1}$ by the dilutions above). After the indicated amount of time, mice were anesthetized with isoflurane and killed by decapitation. Brains were removed and hemisected along the midsagittal plane, and each half was then flash frozen in liquid N $_2$. One half of the brain was prepared as described above for protein analysis, and the other half was used for metabolite analysis. The selective inhibition of FAAH by URB597 was achieved in a manner similar to that described above, except URB597 was dissolved by sonication into 18:1:1 v/v/v saline/emulphor/ethanol (1 mg ml $^{-1}$) and administered intraperitoneally at a volume of 10 μ l g $^{-1}$ weight (10 mg kg $^{-1}$ final dose). Oral administration was performed exactly as described for intraperitoneal administration, except that the vehicle was PEG300.

Measurement of brain lipids. Brain lipid measurements were determined using a previously described procedure³⁵ as detailed in the **Supplementary Methods**.

ABPP-MudPIT analysis of serine hydrolases targeted by JZL184 in vivo. ABPP-MudPIT studies were performed following previously described methods^{27,32} as detailed in the **Supplementary Methods**.

Behavioral studies. Mice were evaluated in the tetrad test for cannabinoid effects and the acetic acid-induced stretching assay as detailed in the **Supplementary Methods**. Animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committees of The Scripps Research Institute and Virginia Commonwealth University.

Note: [Supplementary information](#) and [chemical compound information](#) is available on the [Nature Chemical Biology website](#).

ACKNOWLEDGMENTS

We thank the Cravatt lab for helpful discussion and critical reading of the manuscript. This work was supported by the US National Institutes of Health (DA017259, DA025285, DA007027, DA005274 AA014619, DA024194 and AA06420), the Helen L. Dorris Institute Child and Adolescent Neuro-Psychiatric Disorder Institute and the Skaggs Institute for Chemical Biology.

AUTHOR CONTRIBUTIONS

J.Z.L., L.H.P., A.H.L. and B.F.C. designed the experiments. J.Z.L. and W.L. synthesized and characterized the inhibitors. F.J.P., A.M.S. and L.H.P. measured extracellular endocannabinoid levels. L.B., J.J.B., S.G.K., J.E.S., D.E.S. and A.H.L. performed behavioral studies. J.Z.L. and B.F.C. wrote the manuscript.

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